Bonn zoological Bulletin 69 (1): 11–26
2020 · Filipiak A. et al.
https://doi.org/10.20363/BZB-2020.69.1.011

ISSN 2190–7307
http://www.zoologicalbulletin.de

Research article

urn:lsid:zoobank.org:pub:237955E5-9C3A-4222-8256-EEF0F80BF0F7

Helminths associated with terrestrial slugs in some parts of Europe

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Abstract. A survey of helminths associated with terrestrial slugs focusing on the invasive *Arion vulgaris* and the native *A. ater* was conducted on populations from France, Germany, Netherlands, Norway and Poland. In total, 648 terrestrial slugs were collected from 18 sample sites, and identified by means of morphological examination, dissection of genitalia and molecular analysis using mitochondrial DNA. In addition to *A. vulgaris* and *A. ater*, also *A. vulgaris/A. rufus* hybrids and *A. ater/A. rufus* hybrids were collected. Helminth species were identified based on morphological features and sequencing of the 18S and ITS rDNA regions. The parasites included four nematode species: *Alloionema appendiculatum*, *Angiostoma* sp., *Phasmarhabditis hermaphrodita*, *Entomelas* sp., two trematode species: *Brachylaima mesostoma, Eurytrema* sp., and one cestode (tapeworm) species: *Skrjabinia* sp. *Alloionema appendiculatum* was the most common helminth in the investigated slug populations. Furthermore, we found higher prevalence of trematodes in the invasive *A. vulgaris* compared with the native *A. ater*, while differences in the prevalence for nematodes were not as clear.

Keywords. Slugs, Arionidae, helminth parasites, nematodes, trematodes, tapeworm.

INTRODUCTION

Parasitism plays an important role in the ecology and evolution of terrestrial gastropods (slugs and snails) influencing the evolution of sexual reproduction, life-history traits as well as host resistance leading to host-parasite co-evolution. Parasites have a direct impact on life cycles of their hosts, and the effects of parasites can be modulated by environmental factors. Several studies have been conducted on the impact of climate change on crop pests in relation to natural enemies such as insect predators, parasitoids, pathogenic microorganisms, and helminth parasites (Gerard et al. 2013; Wilson et al. 2015). The presence of helminths in slugs can be influenced by the size, age and the spatial isolation of the host population and by habitat characteristics (Baur & Baur 2005; Gerard et al. 2013; Wilson et al., 2012; Wilson et al. 2015).

Different groups of helminths can be associated with terrestrial gastropods, but slugs as hosts have been given most attention (Ross et al. 2010a; Ross et al. 2010b; Ross et al. 2016). Currently more than 25,000 species of nematodes have been described, of which around 3,500 are parasitic nematodes of invertebrates (Laznik et al. 2010). Nematodes parasitise both slugs and snails, however slugs are parasitised more frequently and by a greater diversity of nematodes than snails (Mengert 1953). This is because slugs usually inhabit soil, thus increasing their exposure to nematodes (Morand et al. 2004; Ross et al. 2010a). Currently, representatives of eight nematode families are known to be associated with terrestrial slugs: Agfidae, Alaninematidae, Alloionematidae, Angiostomatidae, Cosmocercidae, Diplogasteridae, Mermithidae, and Rhabditidae (particularly the genus *Phasmarhabditis*) (Ross et al. 2017). These families are known to form a number of different relationships with slugs, including parasitic (specialist or generalist), phoretic and necromenic associations (Ross et al. 2010b; Ross et al. 2017). Moreover, trematodes, mites, sporozoa, ciliates, and cestodes have also been described to interact with slugs (Stephenson & Knutson 1966; Baur & Baur 2005).

Received: 10.10.2019
Accepted: 14.01.2020

Corresponding editor: B. Huber
Published: 20.02.2020

Terrestrial gastropods are considered to be one of the most successful and diverse animal groups in terrestrial ecosystems (Barker 2001). Many of them have become invasive species in the context of expanding their range of distribution and generating economic damages (e.g., *Lissachatina fulica* Bowdich, 1822 and *Deroceras reticulatum* Müller, 1774) (Hammond & Byers 2002; Ross et al. 2010a). One of the 100 most invasive species in Europe is *Arion vulgaris* Moquin-Tandon, 1855, commonly known as the Spanish slug. It has probably been unintentionally introduced into new habitats via plant matter, packaging, and waste materials (Kozłowski 2007; Hatteland et al. 2013; Zając et al. 2017). It is a major defoliator of plants and causes severe damage in orchards and gardens as well as in crops (Gren et al. 2009). This slug has been assumed to originate from the Iberian Peninsula and spread into Central Europe in the 1950s (Frank et al. 2002), although recent studies suggest a more northern origin, possibly in France (Hatteland et al. 2015; Zemanova et al. 2016). Monitoring the spread of *A. vulgaris* is difficult because the pest is morphologically similar to the other closely related, large arionids (*A. ater* Linnaeus, 1758; *A. rufus* Linnaeus, 1758; *A. magnus* Torres Minguez, 1923; *A. lusitanicus* Mabille, 1868; *A. flagellus* Collinge, 1893) that occur in Europe. *Arion ater*, *A. rufus* and *A. vulgaris* can hybridise with each other (Roth et al. 2012; Dreijers et al. 2013) and introgression has readily been shown, especially between *A. ater* and *A. rufus* (Hatteland et al. 2015; Zemanova et al. 2017). Moreover, *A. vulgaris* may outcompete native slug species because of its large size and high population densities (Frank 2003). Native slugs like *A. ater* and *A. rufus* have been observed to decline and/or disappear in areas colonized by invasive slugs such as *A. vulgaris* in continental Europe (Falkner 1990) and Scandinavia (B. A. Hatteland pers. obs.) and a similar pattern seems to occur where *A. flagellus* has been introduced in Britain (Davies 1987).

There are numerous hypotheses regarding what makes species invasive. Release from natural enemies is regarded to be an important factor supporting invasiveness by many organisms (Torchin et al. 2003). The enemy release hypothesis (ERH) states that the lack of natural enemies in an invader's introduced range influences its abundance or impact (e.g., estimated using individual size, population abundance, or propensity to displace native species) (Torchin et al. 2003; Colautti et al. 2004). Torchin et al. (2003) studied parasite burdens in 26 species of invasive animals and found that most of them had fewer parasites in their introduced areas compared with their home ranges. Indeed, it is less likely that hosts will spread parasites into their introduced range since introduced populations often originate from relatively small subsets of native populations (and sometimes from uninfected life-history stages) (Torchin et al. 2003). However, recent studies have shown that *A. vulgaris* hosts a range of parasites in introduced areas as well as relatively high parasitic loads compared with other native slug species (Ross et al. 2010a; Ross et al. 2016).

This study describes results on the diversity and distribution of helminths associated with *A. vulgaris* and *A. ater* in Europe, i.e., France, Germany, Netherlands, Norway and Poland.

MATERIAL AND METHODS

Collection and identification of slugs and helminths

Slugs were collected from 18 sites in Europe (France, Germany, Netherlands, Norway, Poland) in 2015 and 2016 from late August to October (Table 1). Sites were selected based on information from local growers and gardeners as well as advisory services in the region regarding the presence of slugs. At each site more than 10 slugs were collected and sent to the Institute of Environmental Sciences (Jagiellonian University, Kraków, Poland) and Norwegian Institute of Bioeconomy Research (NIBIO, Ås, Norway). Slugs were identified by means of morphological examination guided by von Proschwitz (2009), dissection of their genitalia and molecular analysis using a fragment of mitochondrial cytochrome c oxidase subunit I (COI, mtDNA). The main features of the genitalia in *A. vulgaris* are the small atrium, almost symmetrical, one-partite, bursa copulatrix oval and a free oviduct with a short, thin posterior end and a thick, rapidly expanding anterior end with a large, asymmetric ligula inside (Wiktor 2004). The main characteristics of *A. ater* are the atrium and vagina considerably narrower than spermatheca, oviduct narrow and the spermatheca spherical (Welter-Schultes 2012). During the dissection of slugs, a piece of tissue was taken for DNA extraction and preserved in 96% ethanol at -80°C.

Slugs were checked for potential helminths, which were identified using a combination of morphological and molecular techniques. The helminths were first classified morphologically as nematodes or trematodes under a stereomicroscope Olympus SZX10. For purposes of molecular identification, all helminth samples (i.e., adults, juveniles and cysts) were transferred to Eppendorf tubes containing 70% ethanol. Each helminth was introduced to a separate Eppendorf tube.

DNA isolation, amplification and sequencing

DNA extraction of slugs was performed using a commercial DNA extraction kit (NucleoSpin® Tissue, Macherey-Nagel, Düren, Germany), which uses a proteinase K to digest proteins within cell membranes and columns with silica membranes and buffers for cleaning extracted DNA. PCR reactions were performed to obtain a fragment of mitochondrial DNA with LCO1490/HC02198 primers (Folmer et al. 1994). A PCR reaction was per-

formed in a reaction mixture of 20 µl per each sample and consisted of 3 µl of template DNA, 0.6 µl of each primer, 2 µl of 10× buffer, 13 µl of ddH2O, 0.6 µl of 20 mM dNTP and 0.2 µl of DreamTaq™ DNA Polymerase (Thermo Fisher Scientific Inc., MA, USA). PCR conditions included 5 min initial denaturation at 94°C and then 1 min denaturation at 94°C, 1 min 30 s annealing at 45°C and 1 min 30 s elongation at 72°C for 5 cycles and then 1 min denaturation at 94°C, 1 min 30 s annealing at 50°C and 1 min elongation at 72°C for 35 cycles followed by a final elongation step for 5 min at 72°C. A volume of 5 µl sample of PCR product was run on a 1.0% agarose gel for 30 min at 100 V to check DNA quality. PCR products were cleaned up by commercial kit (NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel, Düren, Germany). The sequencing reaction was performed in a reaction mixture of 10 µl per each sample and consisted of 2 µl of template DNA, 5.85 µl of ddH2O, 0.15 µl of primer, 1 µl of 5× buffer and 1 µl of Terminator (BrightDye® Terminator Cycle Sequencing Kit, MCLAB, South San Francisco, USA). Sequencing products were cleaned by using a kit to remove terminators after sequencing reactions (ExTerminator, A&A Biotechnology, Gdynia, Poland). The sequencing reactions were performed in Molecular Ecology Lab, Institute of Environmental Sciences (Jagiellonian University, Kraków, Poland).

The genomic DNA of helminths was isolated with a QIAamp DNA Micro Kit (Qiagen, Hilden, Germany) according to the protocol provided by the manufacturer. DNA concentration and its purity were measured using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Polymerase chain reactions (PCR) were performed on the partial 18S and ITS rDNA regions. For nematodes, the ITS region was amplified using two sets of primers, i.e. 18S/26S (Vrain et al., 1992), and N93/N94 (Nadler et al. 2005). Amplification of the 18S region was done with 24F/18P primers (Blaxter et al. 1998). DNA of trematodes and cestodes was amplified using 3S/A28 primers for ITS2 region (Bowles et al. 1995). PCR were performed in a reaction mixture of 20 µl per each sample and consisted of 1 µl of template DNA (100 ng of template DNA in 1 µl volume), 1 µl of each forward and reverse primer (0.5 µM), 10 µl of 2× DreamTaq™ Master Mix (Thermo Fisher Scientific Inc., MA, USA) and 7 µl of sterile distilled water. The PCR amplifications were performed as described in Nermut' et al. (2015) for the 18S/26S primers, Nadler et al. (2005) for N93/N94 primers, Ross et al. (2010b) for 24F/18P, and Prasad et al. (2007) for 3S/A28. A volume of 5 µl sample of PCR product was run on a 1.5% agarose gel for 30 min at 100 V to check DNA quality. For rDNA partial sequences, PCR products were sequenced with primers used for PCR reactions. Sequencing was performed by Genomed (Warsaw, Poland). Contigs assembled were determined using BioEdit version 7.1.3.0 (Hall 1999).

Phylogenetic analysis

The molecular phylogenetic status of helminths was determined using BioEdit version 7.1.3.0 for multiple sequence alignments (Hall, 1999). Multiple nucleotide sequence alignments were generated using also other sequences deposited in GenBank showing the highest similarity to the sequence of examined helminths (Appendix I). The species names and GenBank accession numbers of the sequences compared to the analyzed helminths are shown in the phylograms. Phylogenetic trees were generated using MEGA X (Kumar et al. 2018) by maximum likelihood (ML) and neighbour-joining (NJ) algorithms. The base substitution model was determined for the 18S and ITS using MEGA X under the Bayesian Information Criterion. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Bootstrap values above 60% were considered. Branch lengths indicate evolutionary rates expressed as the number of base differences per site.

Statistical analyses of prevalence

A comparison of parasite prevalence was carried out between the invasive *A. vulgaris* and the native *A. ater*. Hybrid slugs were not included since the number of specimens was low. Generalized linear models (GLMs) with binomial distribution were used to test possible differences in presence/absence of nematodes and trematodes, respectively. All specimens from all investigated populations were included in the data set. Locality was used as an explanatory factor in addition to slug species to test the prevalence of the two different parasite groups. Statistical analyses were performed in the software R (R Core Team 2017).

RESULTS

Slug identification

All collected slugs were identified based on morphology and genital morphology and sequences of cytochrome c oxidase subunit I (COI) based on BLAST search results. In total, 20 European populations were investigated, which included 13 populations of *A. vulgaris* (Norway – 9, Poland – 2, France – 1, Germany – 1), and five populations of *A. ater* (all in Norway). Additionally, two populations of hybrids were collected, one population of *A. ater*/*A. rufus* (Klepp, Norway) and one population of *A. vulgaris*/*A. rufus* (Zoetermeer, Netherlands). From these sites a total of 648 slugs of the genus *Arion* were collected comprising 490 *A. vulgaris,* 92 *A. ater*, 44 *A. vulgaris/A. rufus* hybrids and 22 *A. ater/A. rufus* hybrids (Table 1).

Helminth identification and prevalence

Helminths were found associated with slugs at 17 of the 18 sample sites in Europe (94.4%). A total of 501 (77.3%) of 648 examined slugs were infected with helminths. All slug taxa were infected with nematodes. Trematodes were found in *A. vulgaris* and *A. ater*, and cestodes in *A. vulgaris* and *A. ater/A. rufus* hybrids. In *A. vulgaris*, 359 specimens were infected with helminths, i.e., 148 with nematodes (*Alloionema appendiculatum* Schneider, 1859; *Angiostoma* sp.; *Phasmarhabditis hermaphrodita* Schneider, 1859), 198 with trematodes (*Brachylaiama mesostoma* Rudolphi, 1803) and 13 with cestodes (*Skrjabinia* sp.). In *A. ater,* 74 of the 92 dissected specimens were infected with helminths, i.e., 57 with nematodes (*A. appendiculatum*, *P. hermaphrodita*, *Angiostoma* sp. and *Entomelas* sp.), and 17 with trematodes (*B. mesostoma*, *Eurytrema* sp.). In *A. vulgaris/A. rufus*, 43 of the 44 dissected slugs were infected with nematodes (*A. appendiculatum*). In the 22 dissected *A. ater/A. rufus*, 25 helminths were found, i.e., 23 nematodes (*A. appendiculatum*, *P. hermaphrodita*), and 2 cestodes (*Skrjabinia* sp.) (Table 1).

Nematodes were found in slugs at 14 of the 18 visited sites (Norway – 11, France – 1, Netherlands – 1, Poland – 1; 77.8% of all sample sites), trematodes at 12 of the 18 sites (Norway – 11, France – 1; 66.7% of all sample sites), and cestodes at five of the 18 sites (Norway – 4, Germany – 1; 27.8% of all sample sites). A total of four nematode species (*A. appendiculatum*, *Angiostoma* sp., *P. hermaphrodita*, *Entomelas* sp.), two trematode species (*B. mesostoma, Eurytrema* sp.) and one cestode (*Skrjabinia* sp.) species were identified based on morphological and molecular identification (Table 1; explanation regarding the three species *Entomelas* sp., *Eurytrema* sp., and *Skrjabinia* sp. being listed as "genus sp." is provided in the paragraph "Phylogenetic analysis" below).

From sites positive for nematodes, *A. appendiculatum* was recorded in 11 sites, *P. hermaphrodita* in eight sites, *Angiostoma* sp. in five sites, and *Entomelas* sp. in only one site. For sites positive for trematodes, *B. mesostoma* was found in 11 sites, and *Eurytrema* sp. in only one site. All cestodes were identified as *Skrjabinia* sp. (Table 1).

Phylogenetic analysis

In total, 501 sequences of the 18S and ITS rDNA regions were generated (Table 1). These sequences represented seven species of helminths from seven families. Sequences of the same species were identical across the 18S and ITS rDNA regions, so only one representative sequence was submitted for each taxon. Obtained sequences of helminth species have been deposited in GenBank with the following accession numbers: KY355082–KY355088 (Table 2).

BLAST search for three species sequences did not show exact matches with other sequences deposited in GenBank. The sequence of KY355086 revealed 92.98% identity with *Entomelas dujardini* Maupas, 1916 (accession number: KF999591), sequence of KY355087 revealed 90.46% identity with *Eurytrema pancreaticum* Janson, 1889 (accession number: KY490000), and sequence of KY355088 revealed 83.63% identity with *Skrjabinia cesticillus* Molin, 1858 (accession number: AY382321). Other sequences were characterised by very low query coverage, resulting in low total scores of BLAST searches. None of the sequences available in GenBank were significantly similar to sequences obtained in this study for these three species. Therefore, these three detected helminths (i.e., *Entomelas* sp., *Eurytrema* sp., and *Skrjabinia* sp.) are listed in the study as "genus sp."

Trees that were inferred from maximum-likelihood (ML) and neighbor joining (NJ) revealed identical topologies. Thus only maximum-likelihood results are presented along with bootstrap support from each method of analysis (Figs 1–6). The molecular phylogenetic trees, generated from partial 18S and ITS of rDNA regions with ML and NJ algorithms, showed that the detected nematodes belonged to the species *A. appendiculatum* (Fig. 1) and *P. hermaphrodita* (Fig. 2), and to the genus *Angiostoma* sp., and *Entomelas* sp. The molecular phylogenetic analysis revealed that these two nematodes are closely related to *Angiostoma margaretae* and *A. norvegicum* (Fig. 3), and *Entomelas dujardini* (Fig. 4). BLAST search for *Angiostoma* sequences revealed 100% identity with *Angiostoma margaretae* Ross, Malan, Ivanova, 2011 (accession number: HQ115062) and *Angiostoma norvegicum* Ross, Haukeland, Hatteland, Ivanova, 2017 (accession number: KU712560). Due to the fact that not enough material was available for molecular work, the 28S and COI could not be used for the identification of these nematodes. Therefore, the nematodes were listed as *Angiostoma* sp. In the study of Singh et al. (2019), partial 18S and D2D3 sequences from the same DNA material were also found to be almost 100% similar to sequences from two different species. The evolutionary history of *A. appendiculatum* was inferred by using the ML method based on the General Time Reversible (GTR) model, and *P. hermaphrodita*, *Angiostoma* sp. and *Entomelas* sp. based on the Tamura 3-parameter model.

The molecular phylogenetic trees, generated from partial ITS of rDNA regions with ML and NJ algorithms, showed that the detected trematodes belong to the species *B. mesostoma* and to the genus *Eurytrema* sp. (Fig. 5). The molecular phylogenetic analysis revealed that this trematode is closely related to *E. pancreaticum.* The ML method based on the GTR model indicate the evolutionary history of *B. mesostoma* and *Eurytrema* sp. The molecular phylogenetic trees, generated from partial ITS of rDNA regions with ML and NJ algorithms,

Table 1. The prevalence (%) of infection of terrestrial slugs with parasite species collected in Europe (N = number of slugs examined).

Locality	GPS coordinates	Slug species	N	Parasite species	%
France:					
Metz	49°7’1’’N 6°10’59’’E	*Arion vulgaris*	20	*Alloionema appendiculatum* *Brachylaima mesostoma*	65 15
Germany:					
Gauting	48°4’1’’N 11°22’1’’E	*Arion vulgaris*	20	*Skrjabinia* sp.	40
Netherlands:					
Zoetermeer	52°03’54’’N 4°30’31’’E	*Arion vulgaris/* *A. rufus*	44	*Alloionema appendiculatum*	97.7
Norway:					
Balestrand	61°10’39’’N 6°24’14’’E	*Arion ater*	20	*Alloionema appendiculatum* *Brachylaima mesostoma* *Phasmarhabditis hermaphrodita*	45 20 10
Hana, Sandnes	69°31’1’’N 20°22’1’’E	*Arion ater*	19	*Alloionema appendiculatum* *Eurytrema* sp. *Phasmarhabditis hermaphrodita*	100 5.3 36.8
Horten	59°25’1’’N 10°25’59’’E	*Arion vulgaris*	112	*Alloionema appendiculatum* *Brachylaima mesostoma* *Phasmarhabditis hermaphrodita* *Skrjabinia* sp.	5.4 87.5 0.9 1.8
Jensvoll, Bodø	67°16’5’’N 14°24’0’’E	*Arion vulgaris*	20	*Brachylaima mesostoma*	25
Kjenneveien, Fredrikstad	59°14’59’’N 10°51’30’’E	*Arion ater*	30	*Angiostoma* sp. *Brachylaima mesostoma* *Entomelas* sp.	13.3 40 3.3
Kristiansand	58°10’1’’N 8°0’0’’E	*Arion vulgaris*	30	*Alloionema appendiculatum* *Brachylaima mesostoma*	6.7 100
Oslo	59°57’41’’N 10°53’13’’E	*Arion vulgaris*	25	*Angiostoma* sp. *Phasmarhabditis hermaphrodita*	16 16
Lindhjem, Larvik	59°0’42’’N 9°58’34’’E	*Arion vulgaris*	39	*Alloionema appendiculatum* *Angiostoma* sp. *Brachylaima mesostoma* *Skrjabinia* sp.	5.1 7.7 33.3 5.1
Manstad, Fredrikstad	59°16’9’’N 10°46’7’’E	*Arion vulgaris*	24	*Brachylaima mesostoma* *Skrjabinia* sp.	50 4.2
Klepp	58°46’59’’N 5°35’60’’E	*Arion vulgaris*	29	*Alloionema appendiculatum* *Brachylaima mesostoma* *Phasmarhabditis hermaphrodita*	48.3 51.7 3.4
		Arion ater/A. rufus	22	*Alloionema appendiculatum* *Phasmarhabditis hermaphrodita* *Skrjabinia* sp.	95.5 9.1 9.1
Vesterøya, Sandefjord	59°6’14’’N 10°14’34’’E	*Arion ater*	13	*Angiostoma* sp. *Phasmarhabditis hermaphrodita*	61.5 23.1
Bergen	60°23’37’’N 5°22’38’’E	*Arion ater*	10	*Alloionema appendiculatum* *Angiostoma* sp. *Phasmarhabditis hermaphrodita*	10 10 20
		Arion vulgaris	26	*Alloionema appendiculatum* *Angiostoma* sp. *Brachylaima mesostoma* *Phasmarhabditis hermaphrodita*	38.5 80.8 38.5 50
Time, Bryne	58°43’59’’N 5°39’0’’E	*Arion vulgaris*	25	*Alloionema appendiculatum* *Brachylaima mesostoma* *Phasmarhabditis hermaphrodita*	80 48 8
Poland:					
Igołomia	50°5’20’’N 20°14’20’’E	*Arion vulgaris*	100	*Alloionema appendiculatum*	32
Rzeszów	50°1’59’’N 22°0’18’’E	*Arion vulgaris*	20		–

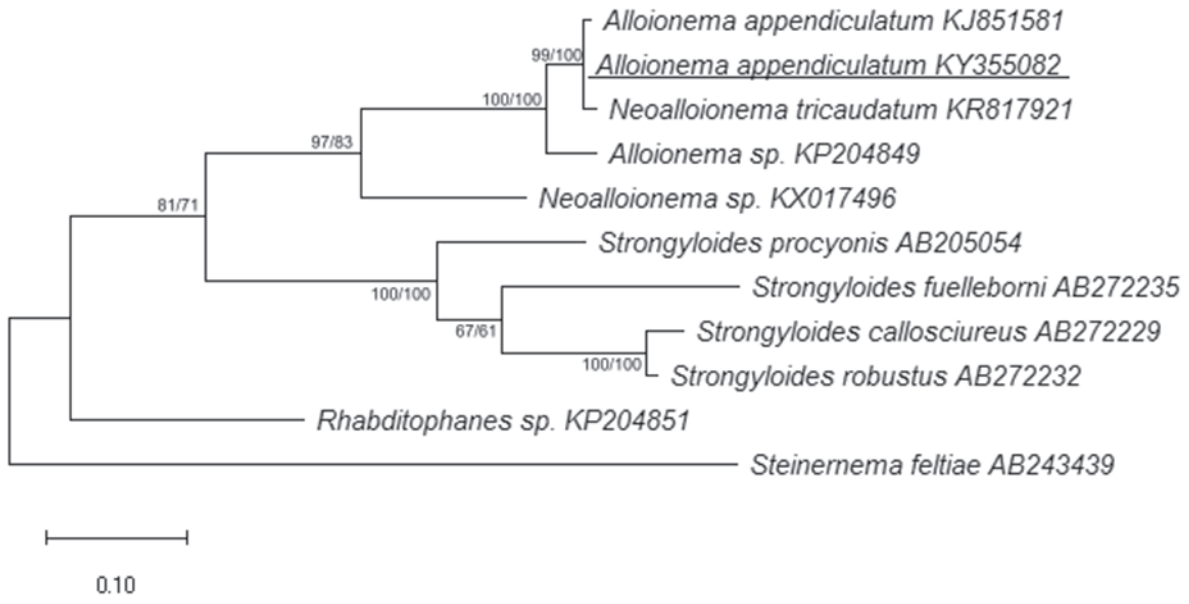

Fig. 1. Unrooted maximum likelihood phylogeny of ITS rDNA regions for *Alloionema appendiculatum*. The scale bar represents 0.10 substitutions per nucleotide position. Only bootstrap values above 60% are shown.

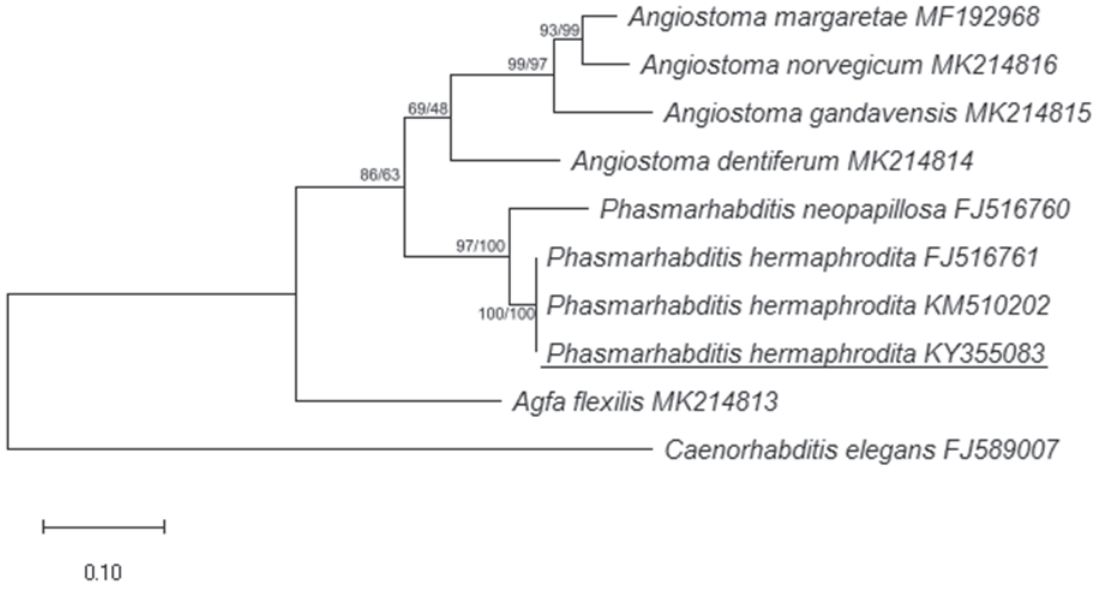

Fig. 2. Unrooted maximum likelihood phylogeny of ITS rDNA regions for *Phasmarhabditis hermaphrodita.* The scale bar represents 0.10 substitutions per nucleotide position. Only bootstrap values above 60% are shown.

showed that the detected cestodes belong to the genus *Skrjabinia* sp. (Fig. 6). The molecular phylogenetic analysis (ML method based on the Hasegawa-Kishino-Yano model) revealed that this cestode is most closely related to *S. cesticillus* and *Raillietina echinobothrida.*

Statistical analyses of prevalence

A tendency of higher prevalence of trematodes was found in *A. vulgaris* populations compared with *A. ater* populations (Fig. 7; GLM, p=0.0618). On the other hand, a

Table 2. The accession numbers of examined slug-parasites with NCBI matches.

Parasite family/species	GenBank no.	NCBI match	Query coverage	Percentage identity	Source
Family: Alloionematidae *Alloionema appendiculatum*	KY355082	*Alloionema appendiculatum*; KJ851581	100	98.95	Nermut' *et al.* (2015)
Family: Rhabditidae *Phasmarhabditis hermaphrodita*	KY355083	*Phasmarhabditis hermaphrodita*; FJ516761	100	100	–
Family: *Angiostomatidae* *Angiostoma* sp.	KY355084	*Angiostoma margaretae*; HQ115062	100	100	Ross *et al.* (2011)
		Angiostoma norvegicum; KU712560	100	100	Ross *et al.* (2017)
Family: Rhabdiasidae *Entomelas* sp	KY355086	*Entomelas dujardini*; KF999591	100	92.98	Tkach *et al.* (2014)
Family: Brachylaimidae *Brachylaima mesostoma*	KY355085	*Brachylaima mesostoma*; KT074964	100	100	Heneberg *et al.* (2016)
Family: Dicrocoeliidae *Eurytrema* sp.	KY355087	*Eurytrema pancreaticum;* KY490000	99	90.46	Su *et al.* (2018)
Family: Davaineidae *Skrjabinia* sp.	KY355088	*Skrjabinia cesticillus*; AY382321	81	83.63	–

somewhat lower prevalence of nematodes in *A. vulgaris* than in *A. ater* (Fig. 8) was revealed, although this difference was not significant (GLM, p=0.1569).

DISCUSSION

This study presents the occurrence of helminths associated with two slug species: *A. vulgaris* and *A. ater*, as well as *A. vulgaris/A. rufus* hybrids, and *A. ater/A. rufus* hybrids in some parts of Europe, i.e., France, Germany, Netherlands, Norway and Poland. Previous studies on the diversity and distribution of slug parasites have focused on the presence of nematodes in slugs (Mengert 1953; Gleich et al. 1977; Charwat & Davies 1999; Laznik et al. 2009; Ross et al. 2010b; Ross et al. 2011; Ivanova et al. 2013; Ross et al. 2016; Singh et al. 2019). In our study, a total of seven species of helminths were found to be associated with these slugs including nematodes, trematodes and one cestode species. The nematodes were identified to *Alloionema appendiculatum*, *Angiostoma* sp., *Phasmarhabditis hermaphrodita*, *Entomelas* sp., trematodes were identified as *Brachylaima mesostoma*, *Eurytrema* sp., and the cestode species was identified as *Skrjabinia* sp. We found a tendency for higher prevalence of trematodes in *A. vulgaris* compared with the native *A. ater*. However, we did not find the same pattern in terms of prevalence for nematodes.

Slug-parasitic nematodes were found in all organs of the body cavity and also on foot muscles, typical of *A. appendiculatum*. The most intensively studied species of slug-parasitic nematode of agricultural and horticultural crops is *P. hermaphrodita*. In our survey, *P. hermaphrodita* was found in eight of the 18 sample sites examined, and was found to parasitize both *A. ater*, *A. vulgaris*, as well as *A. ater /A. rufus* hybrids. Only juveniles of *P. hermaphrodita* were detected in our study. Within the Rhabditidae family, *Phasmarhabditis* is the only genus that

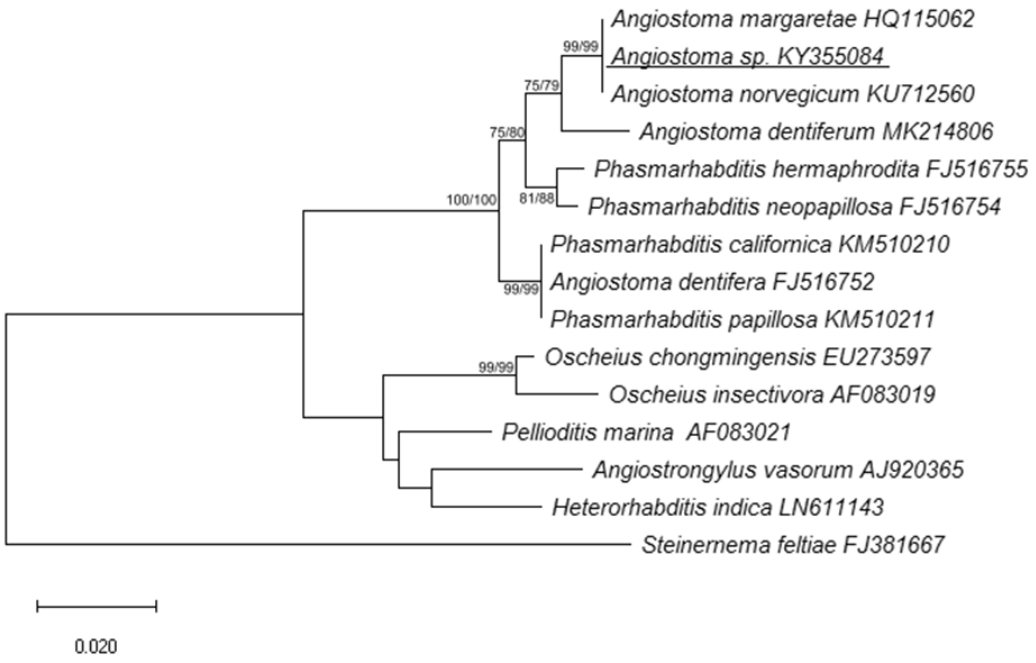

Fig. 3. Unrooted maximum likelihood phylogeny of 18S rDNA regions for *Angiostoma* sp. The scale bar represents 0.020 substitutions per nucleotide position. Only bootstrap values above 60% are shown.

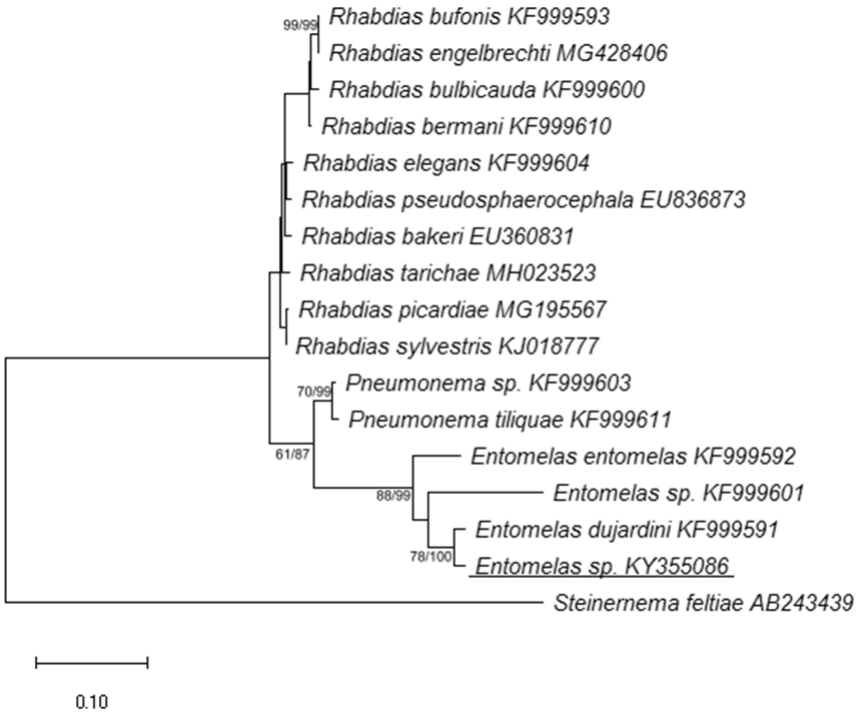

Fig. 4. Unrooted maximum likelihood phylogeny of ITS rDNA regions for *Entomelas* sp. The scale bar represents 0.10 substitutions per nucleotide position. Only bootstrap values above 60% are shown.

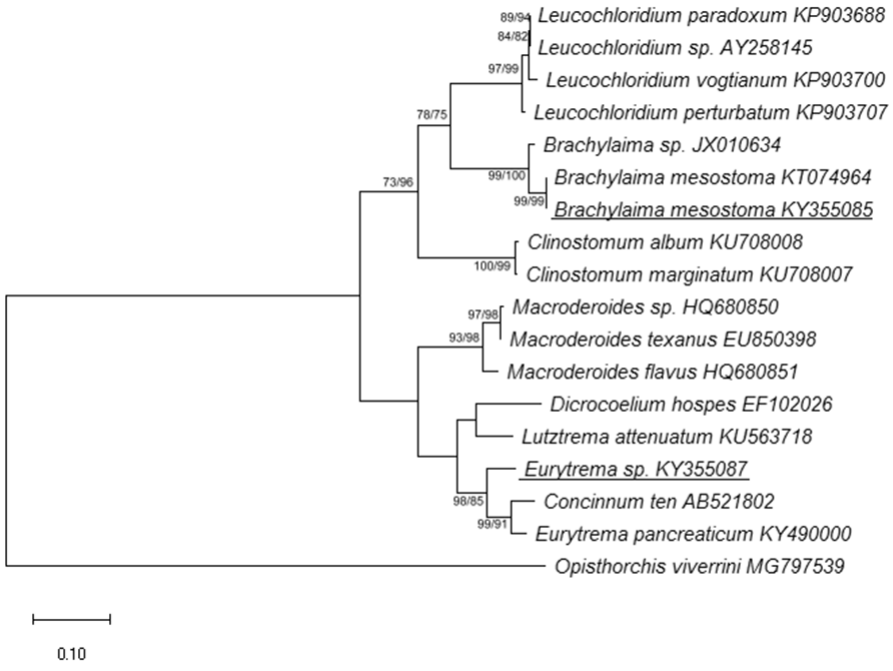

Fig. 5. Unrooted maximum likelihood phylogeny of ITS rDNA regions for *Brachylaima mesostoma* and *Eurytrema* sp. The scale bar represents 0.10 substitutions per nucleotide position. Only bootstrap values above 60% are shown.

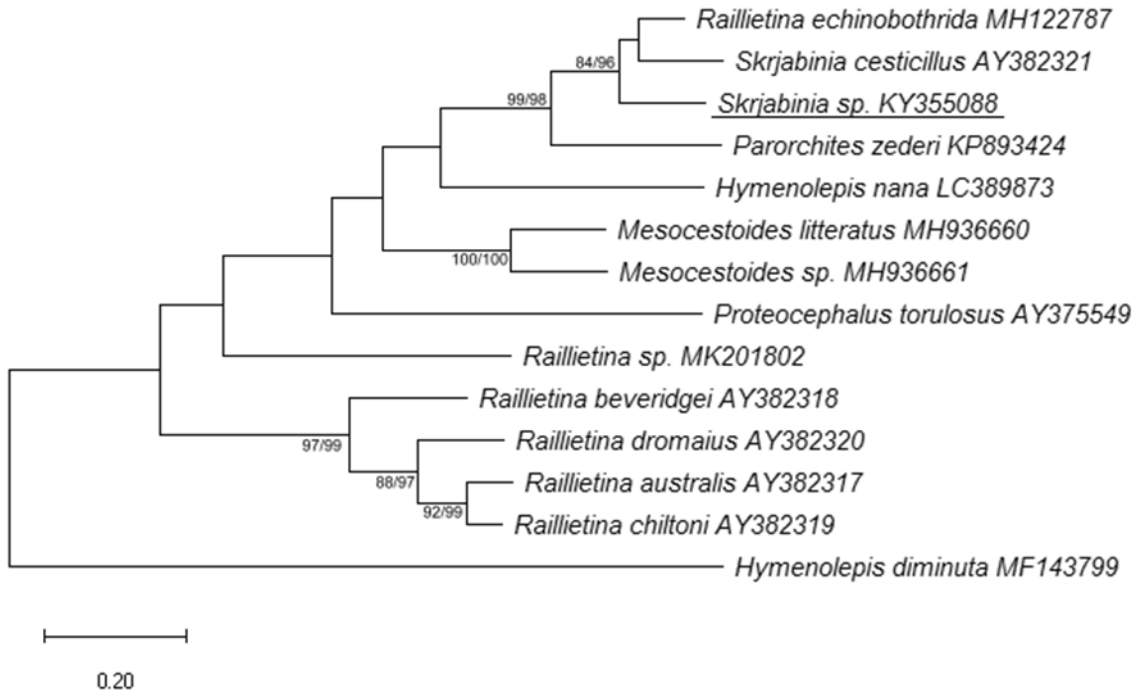

Fig. 6. Unrooted maximum likelihood phylogeny of ITS rDNA regions for *Skrjabinia* sp. The scale bar represents 0.20 substitutions per nucleotide position. Only bootstrap values above 60% are shown.

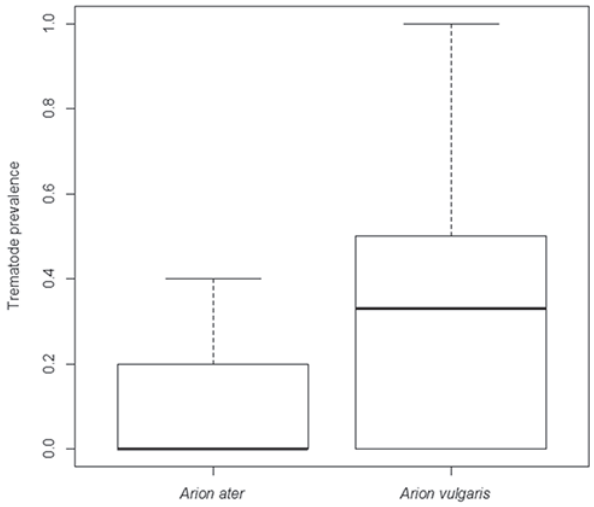

Fig. 7. Boxplot of trematode prevalence (proportionally) in populations of *Arion vulgaris* and *A. ater*.

Fig. 8. Boxplot of nematode prevalence (proportionally) in populations of *Arion vulgaris* and *A. ater*.

is considered to be truly parasitic towards slugs. *P. hermaphrodita* is known to be capable of killing many species of slugs from several families (Morand et al. 2004; Wilson et al. 2012). The nematode has been formulated into an effective biological control agent (Tan & Grewal 2001; Wilson et al. 2015). Infective dauer juveniles (a non-feeding survival stage) of *P. hermaphrodita* seek out the host through a combination of both chemotactic and chemokinetic responses towards chemical attractants in slug feces and mucus from the foot and mantle (Rae et al. 2006; Hapca et al. 2007).

Another nematode known to be associated with terrestrial gastropods is *Alloionema appendiculatum*. This nematode has a broad geographical distribution and has been found in areas including Europe, Australia and North America (Morand et al. 2004; Laznik et al. 2009; Nermut' et al. 2015). In our study, *A. appendiculatum* was found in 11 of the 18 sample sites examined. The nematode was found to parasitize *A. ater*, *A. vulgaris*, and *A. ater /A. rufus* and *A. vulgaris/A. rufus* hybrids. *A. appendiculatum* is a common juvenile parasite of many terrestrial gastropods. This nematode has both parasitic and free-living life stages. During the parasitic cycle, third-stage juveniles (J3) enter the slug's body through its foot, where the nematodes moult to the fourth-stage juvenile (J4) which become encapsulated in the pedal musculature. These juveniles then exit the slug and moult into free-living immature adults (Cabaret & Morand 1990; Laznik et al. 2009). In our study we also identified *Angiostoma* sp., present in five of the 18 sample sites examined. The nematode was found to parasitize both *A. ater* and *A. vulgaris*. With the recent discovery of *A. gandavensis*, the total number of described species of the genus *Angiostoma* is now 19, of which 15 are described from molluscan hosts (Singh et al. 2019). International surveys reveal that molluscan angiostomatids are present in Europe, North America, Africa, South-East Asia and New Zealand (Ivanova & Wilson 2009; Ivanova & Spiridonov 2010; Ross et al. 2010a, b; Ross et al. 2011; Ross et al. 2017).

The other nematode identified in our study was *Entomelas* sp. The nematode was only found in one of the 18 sample sites examined, and only in the native *A. ater*. *Entomelas* sp. is classified in the family Rhabdiasidae, which includes up to 100 known nematode species parasitic in amphibians and reptiles. All share some morphological characters but the most remarkable feature of rhabdiasids is the regular alternation of parasitic and free-living generations (heterogony) in their life cycles (Tkach et al. 2014). *Entomelas dujardini* and *E. entomelas* are commonly associated with *Anguis fragilis*. Experimental infection of the slugs *Deroceras reticulatum* (Agriolimacidae) and *Arion subfuscus* (Arionidae) with infective larvae of *E. entomelas* and *E. dujardini* has revealed that both slug species are classed as paratenic (euparatenic) hosts for these nematode species (Kuzmin & Sharpilo 2000).

Among the detected parasites were also trematodes, i.e., *B. mesostoma* and *Eurytrema* sp. We found *B. mesostoma* in 11 of the 18 sample sites examined and it was found in both *A. ater* and *A. vulgaris*. The genus *Brachylaima* contains 72 species that parasitize mammals and birds as definitive hosts around the world, except Antarctica. Terrestrial gastropods are involved as first and second intermediate hosts. They are important from a public health point of view, as they cause diseases

in humans like hemorrhagic enteritis, diarrhea, inflammation of the bile ducts, and anemia (Sirgel et al. 2012; Suleman & Khan 2016; Valente et al. 2016).

The trematode *Eurytrema* sp. was found in one of the 18 sample sites, in *A. ater* only. Species of *Eurytrema* are natural parasites of domestic animals (e.g., cattle, goats, sheep, pigs, dogs) and wild ruminants (such as buffalos, camels, deer) as well as monkeys and humans parasitizing pancreatic ducts and bile ducts. Rarely, terrestrial snails of various species (e.g., *Bradybaena similaris*) are intermediate hosts for these parasites (Pinheiro & Amato 1994). These parasites often cause epithelial hyperplasia, hypertrophy of pancreatic ducts, and periductal fibrosis that lead to eurytrematosis (Cai et al. 2012; Manga-González & Ferreras 2014).

Terrestrial slugs can also be associated with tapeworms. One of the most common tapeworm affecting poultry systems is *Davainea proglottina* and the intermediate hosts are gastropods. Tapeworm segments that pass through poultry feces are ingested by snails and slugs (of the genera *Agriolimax*, *Arion*, *Cepaea* and *Limax*) and within three weeks a cysticercoid is produced. Adult tapeworms are produced in the infected host 8-15 days after ingestion of an infected snail or slug (Jordan & Pattison 1996).

In our study, the tapeworm *Skrjabinia* sp. was found in five of the 18 sample sites examined. The tapeworm was found in *A. vulgaris* and *A. ater/A. rufus* hybrids. *Skrjabinia* is a genus of tapeworms that includes helminth parasites of vertebrates, mostly of birds. One of the most common parasitic platyhelminths in modern poultry facilities throughout the world is *S. cesticillus*. It is a relatively harmless species among intestinal cestodes in spite of a high prevalence. Sometimes called "broad-headed tapeworm", it infects the small intestine of chicken and occasionally other birds, such as guinea fowl and turkey, which are generally in close proximity to backyard poultry (Kaufmann 1996; Morishita & Schaul 2007). Our study showed that, in some cases, a single slug was infected with up to two different species of parasites (i.e., by two species of nematodes or by one species of nematode and one species of trematode). Two different parasites detected from one host was also sometimes observed in a study by Singh et al. (2019). Moreover, our study confirmed that endoparasitic helminths appear to have a broad host range of slug species (Ross et al. 2010b; Singh et al. 2019). *Alloionema appendiculatum* was reported from all four different slug species, *P. hermaphrodita* from three, *Angiostoma* sp. from two, *B. mesostoma* from two, and *Skrjabinia* sp. also from two.

The enemy release hypothesis suggests that species become invasive due to a lack of enemies in their introduced areas (Mitchell & Power 2003; Torchin et al. 2003). However, Colautti et al. (2004) suggested that there are strong, enemy-specific effects on host survival and that hosts have developed tailored defenses. Thus, one may expect that it is the release from specific enemies that causes direct changes to survivorship, fecundity, biomass, or demographic variables that really matters. Alternatively, the loss of enemies against which a host is well defended would be of little consequence for the host populations (Colautti et al. 2004). We found a contrasting pattern in our study, suggesting that the prevalence of trematodes in invasive slugs is higher than in native species. The role of trematodes in slugs should be further investigated including comparative studies with nematodes.

The anthropogenic spread of slugs may potentially lead to a higher degree of mixing of slug populations than would occur when spread naturally. This may also include the spread of parasites along with their slug hosts, which may have implications for the prevalence of trematodes and cestodes that use slugs as intermediate hosts and thus may become more common in domestic mammals such as cattle and sheep.

Acknowledgements. The authors would like to thank Karin Westrum, Henrik Anztée-Hyllseth, Elisa Baluya Gauslaa (NIBIO, Norway) and Daniel Kübler (Jagiellonian University, Poland) for help in dissections of slugs. The authors would finally like to thank two anonymous reviewers for valuable comments and Bernhard A. Huber (Bonn zoological Bulletin) for useful suggestions during the peer review.

Financial support. This work was supported by the Polish-Norwegian Research Programme (Pol- Nor/201888/77).

REFERENCES

Barker GM (2001) The biology of terrestrial molluscs. CABI Publishing, Wallingford, UK

Baur A, Baur B (2005) Interpopulation variation in the prevalence and intensity of parasitic mite infection in the land snail *Arianta arbustorum.* Invertebrate Biology 124: 194–201

Blaxter ML, de Ley P, Garey JR, Liu LX, Scheldeman P, Vierstraete A, Vanfleteren JR, Mackey LY, Dorris M, Frisse LM, Vida JT, Thomas WK (1998) A molecular evolutionary framework for the phylum Nematoda. Nature 392: 71–75

Bowles J, Blair D, McManus DP (1995) A molecular phylogeny of the human schistosomes. Molecular Phylogenetics and Evolution 4: 103–109

Cabaret J, Morand S (1990) Single and dual infection of the land snail *Helix aspersa* with *Muellerius capillaris* and *Alloionema appendiculatum* (Nematoda). Journal for Parasitology 76: 579–580

Cai Z, Zhang Y, Ye X (2012) Phylogenetic relationships of the genus *Eurytrema* from domestic and wild animal based on 18S rRNA sequences. Parasitology Research 111: 1637–1644

Charwat SM, Davies KA (1999) Laboratory screening of nematodes isolated from South Australia for potential as biocontrol agents of helicid snails. Journal of Invertebrate Pathology 74: 55–61

Colautti RI, Ricciardi A, Grigorovich IA, MacIsaac HJ (2004) Is invasion success explained by the enemy release hypothesis? Ecology Letters 7: 721–733

Davies MJ (1987) *Arion flagellus* Collinge and *A. lusitanicus* Mabille in the British Isles. A morphological, biological and taxonomic investigation. Journal of Conchology 32: 339–354

Dreijers E, Reise H, Hutchinson JMC (2013) Mating of the slugs *Arion lusitanicus* auct. non Mabille and *A. rufus* (L.): Different genitalia and mating behaviours are incomplete barriers to interspecific sperm exchange. Journal of Molluscan Studies 79: 51–63

Falkner G (1990) Binnenmollusken. In: Fechter R & Falkner G (eds) Weichtiere. Europäische Meeres- und Binnenmollusken. Steinbachs Naturführer, Mosaik Verlag, München

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular marine biology and biotechnology 3: 294–299

Frank T (2003) Influence of slug herbivory on the vegetation development in an experimental wildflower strip. Basic and Applied Ecology 4: 139–147

Frank T, Bieri K, Speiser B (2002) Feeding deterrent effect of carvone, a compound from caraway seeds, on the slug *Arion lusitanicus*. Annals of Applied Biology 141: 93–100

Gerard PJ, Barringer JRF, Charles JG, Fowler SV, Kean JM, Phillips CB, Tait AB, Walker GP (2013) Potential effects of climate change on biological control systems: case studies from New Zealand. BioControl 58:149–162

Gleich JG, Gilbert FF, Kutscha NP (1977) Nematodes in terrestrial gastropods from central Maine. Journal of Wildlife Disease 13: 43–46

Gren I-M, Isacs L, Carlsson M (2009) Costs of alien invasive species in Sweden. Ambio 38: 135–140

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98

Hammond RB, Byers RA (2002) Agriolimacid and arionid as pests slugs in conservation tillage soybean and corn cropping in North America. In: Barker GM (ed.) Molluscs as Crop Pests. CABI Publishing

Hapca S, Crawford J, Rae R, Wilson M, Young I (2007) Movement of the parasitic nematode *Phasmarhabditis hermaphrodita* in the presence ofmucus fromthe host slug *Deroceras reticulatum*. Biological Control 41: 223–229

Hatteland BA, Roth S, Andersen A, Kaasa K, Støa B, Solhøy T (2013) Distribution and spread of the invasive slug *Arion vulgaris* Moquin-Tandon in Norway. Fauna norvegica 32: 13–26

Hatteland BA, Solhøy T, Schander C, Skage M, von Proschwitz T, Noble LR (2015) Introgression and differentiation of the invasive slug *Arion vulgaris* from native *A. ater*. Malacologia 58: 303–321

Heneberg P, Sitko J, Bizos J (2016) Molecular and comparative morphological analysis of central European parasitic flatworms of the superfamily Brachylaimoidea Allison, 1943 (Trematoda: Plagiorchiida). Parasitology 143: 455–474

Ivanova ES, Wilson MJ (2009) Two new species of *Angiostoma Dujardin*, 1845 (Nematoda: Angiostomatidae) from British terrestrial molluscs. Systematic Parasitology 74: 113–124

Ivanova ES, Spiridonov SE (2010) *Angiostoma glandicola* sp. n. (Nematoda: Angiostomatidae): a parasite in the land snail *Megaustenia* sp. from the Cat Tien Forest, Vietnam. Journal of Helminthology 84: 297–304

Ivanova ES, Panayotova-Pencheva MS, Spiridonov SE (2013) Observations on the nematode fauna of terrestrial molluscs of the Sofia area (Bulgaria) and the Crimea peninsula (Ukraine). Russian Journal of Nematology 21: 41–49

Jordan FTW, Pattison M (1996) Poultry Diseases. W.B. Saunders Company Ltd, London

Kaufmann J (1996) Parasites of poultry. In: Kaufmann J (ed.) Parasitic Infections of Domestic Animals: A Diagnostic Manual. Birkhäuser Verlag, BaselKozłowski J (2007) The distribution, biology, population dynamics and harmfulness of *Arion lusitanicus* Mabille, 1868 (Gastropoda: Pulmonata: Arionidae) in Poland. Journal of Plant Protection Research 47: 219–230

Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35: 1547–1549

Kuzmin YI, Sharpilo VP (2000) On the paratenic parasitism in the nematodes from the family Rhabdiasidae - parasites of anguid lizards (Anguidae). Vestnik zoologii 34: 99–101

Laznik Ž, Ross JL, Tímea T, Lakatos T, Vidrih M, Trdan S (2009) First record of the nematode *Alloionema appendiculatum* Schneider (Rhabditida: Alloionematidae) in Arionidae sulgs in Slovenia. Russian Journal of Nematology 17: 137–139.

Laznik Ž, Ross JL, Trdan S (2010) Massive occurrence and identification of the nematode *Alloionema appendiculatum* Schneider (Rhabditida: Alloionematidae) found in Arionidae slugs in Slovenia. Acta Agriculturae Slovenica 95: 43–49 .

Manga-González MY, Ferreras MC (2014) Dicrocoeliidae family: major species causing veterinary diseases. Advances in Experimental Medicine and Biology 766: 393–428

Mengert H (1953) Nematoden und Schneken. Zeitschrift für Morphologie und Ökologie Tiere 41: 311–349

Mitchell CE, Power AG (2003) Release of invasive plants from fungal and viral pathogens. Nature 421: 625–627

Morand S, Wilson MJ, Glen DM (2004) Nematodes (Nematoda) parasitic in terrestrial gastropods. Pp. 525–557 in: Barker GM (ed) Natural enemies of terrestrial molluscs. CABI Publishing. Wallingford

Morishita TY, Schaul JC (2007) Parasites of birds. Pp. 217–302 in: Baker DG (ed) Flynn's Parasites of Laboratory Animals (2 ed.). Blackwell Publishing Professional Ames, IowaNadler SA, D'Amelio S, Dailey MD, Paggi L, Siu S, Sakanari JA (2005) Molecular phylogenetics and diagnosis of *Anisakis*, *Pseudoterranova*, and *Contracaecum* from Northern Pacific marine mammals. Journal of Parasitology 91: 1413–1429

Nermut' J, Půža V, Mráček Z (2015) Re-description of the slug-parasitic nematode *Alloionema appendiculatum* Schneider, 1859 (Rhabditida: Alloionematidae). Nematology 17: 897–910

Pinheiro J, Amato SB (1994) *Eurytrema coelomaticum* (Digenea: Dicrocoeliidae): the effects of infection on carbohydrate contents of its intermediate snail host, *Bradybaena similaris* (Gastropoda, Xanthonychidae). Memórias do Instituto Oswaldo Cruz 89: 407–410

Prasad PK, Tandon V, Chatterjee A, Bandyopadhyay S (2007) PCR-based determination of internal transcribed spacer (ITS) regions of ribosomal DNA of giant intestinal fluke, *Fasciolopsis buski* (Lankester, 1857) Looss, 1899. Parasitology Research 101: 1581–1587

R Core Team (2017) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/

Rae RG, Robertson JF, Wilson MJ (2006) The chemotactic response of *Phasmarhabditis hermaphrodita* (Nematoda: Rhabditida) to cues of *Deroceras reticulatum* (Mollusca: Gastropoda). Nematology 8: 197–200

Ross JL, Ivanova ES, Severns PM, Wilson MJ (2010a) The role of parasite release in invasion of the USA by European slugs. Biological Invasions 12: 603–610

Ross JL, Ivanova ES, Spiridonov SE, Waeyenberge L, Moens M, Nicol GW, Wilson MJ (2010b) Molecular phylogeny of slug-parasitic nematodes inferred from 18S rRNA gene sequences. Molecular Phylogenetics and Evolution 55: 738–743

Ross JL, Ivanova ES, Malan AP (2011) *Angiostoma margaretae* n. sp. (Nematoda: Angiostomatidae) a parasite of the milacid slug *Milax gagates* Draparnaud collected near Caledon, South Africa. Systematic Parasitology 79: 71–76

Ross JL, Ivanova ES, Hatteland BA, Brurberg MB, Haukeland S (2016) Survey of nematodes associated with terrestrial slugs in Norway. Journal of Helminthology 90: 583–587

Ross JL, Haukeland S, Hatteland BA, Ivanova ES (2017) *Angiostoma norvegicum* n. sp. (Nematoda: Angiostomatidae) a parasite of arionid slugs in Norway. Systematic Parasitology 94: 51–63

Roth S, Hatteland BA, Solhoy T (2012) Some notes on reproductive biology and mating behaviour of *Arion vulgaris* Moquin-Tandon 1855 in Norway including a mating experiment with a hybrid of *Arion rufus* (Linnaeus 1758) x *ater* (Linnaeus 1758). Journal of Conchology 41: 249–258

Singh PR, Couvreur M, Decraemer W, Bert W (2019) Survey of slug-parasitic nematodes in East and West Flanders, Belgium and description of *Angiostoma gandavensis* n. sp. (Nematoda: Angiostomidae) from arionid slugs. Journal of Helminthology: 1–11

Sirgel WF, Artigas P, Bargues MD, Mas-Coma S (2012) Life cycle of *Renylaima capensis*, a brachylaimid trematode of shrews and slugs in South Africa: two-host and three-host transmission modalities suggested by epizootiology and DNA sequencing. Parasites & Vectors 5: 169. https://doi.org/10.1186/1756-3305-5-169

Stephenson JW, Knutson LV (1966) A resume of recent studies of invertebrates associated with slugs. Journal of Economic Entomology 59: 356–360

Su X, Zhang Y, Zheng X, Wang XX, Li Y, Li Q, Wang CR (2018) Characterization of the complete nuclear ribosomal DNA sequences of *Eurytrema pancreaticum*. Journal of Helminthology 92: 484–490

Suleman S, Khan MS (2016) First report of the genus *Brachylaima* Dujardin, 1843 (Trematoda: Brachylaimidae) from the small intestine of common myna (*Acridotheres tristis*) of district Swabi, Khyber Pakhtunkhwa, Pakistan. Turkish Journal of Zoology 40: 595–600

Tan L, Grewal PS (2001) Infection behavior of the rhabditid nematode *Phasmarhabditis hermaphrodita* to the grey garden slug *Deroceras reticulatum*. Journal of Parasitology 87: 1349–1354

Tkach VV, Halajian A, Kuzmin Y (2014) Phylogenetic affinities and systematic position of *Entomelas sylvestris* Baker, 1982 (Nematoda: Rhabdiasidae), a parasite of *Breviceps sylvestris* FitzSimons (Amphibia: Brevicipitidae) in South Africa. Systematic Parasitology 87: 293–298

Torchin ME, Lafferty KD, Dobson AP, McKenzie VJ, Kuris AM (2003) Introduced species and their missing parasites. Nature 421: 628–630

Valente R, Diaz JD, Salomón OD, Navone GT (2016) The role of *Phyllocaulis variegatus* (Mollusca: Veronicellidae) in the transmission of digenean parasites. Revista Mexicana de Biodiversidad 87: 255–257

von Proschwitz T (2009) Snigel: fridstörare i örtagården: vetenskap och fakta. Småskrifter utgivna av Bohusläns museum. Uddevalla, Sweden: Bohusläns museum

Vrain TC, Wakarchuk DA, Levesque AC, Hamilton RI (1992) Intraspecific rDNA restriction fragment length polymorphism in the *Xiphinema americanum* group. Fundamental and Applied Nematology 15: 563–573

Welter-Schultes FW (2012) European non-marine molluscs, a guide for species identification/Bestimmungsbuch für europäische Land- und Süsswassermollusken. Planet Poster Editions, Göttingen

Wiktor A. (2004) Ślimaki lądowe Polski. Mantis, Olsztyn (In Polish)

Wilson MJ, Burch G, Tourna M, Aalders LT, Barker G (2012) The potential of a New Zealand strain of *Phasmarhabditis hermaphrodita* for biological control of slugs. New Zealand Plant Protection 65: 161–165

Wilson MJ, Digweed AJ, Brown J, Ivanonva ES, Hapca SH (2015) Invasive slug pests and their parasites – temperature responses and potential implications of climate change. Biology and Fertility of Soils 51: 739–748

Zając KS, Gaweł M, Filipiak A, Kramarz P (2017) *Arion vulgaris* Moquin-Tandon, 1855 – the aetiology of an invasive species. Folia Malacologica 25: 81–93

Zemanova MA, Knop E, Heckel G (2016) Phylogeographic past and invasive presence of *Arion* pest slugs in Europe. Molecular Ecology 25, 5747–5764

Zemanova MA, Knop E, Heckel G (2017) Introgressive replacements of natives by invading *Arion* pest slugs. Scientific Reports 7: 14908. https://doi.org/10.1038/s/41598-017-14619-y

APPENDIX

Species name of helminths, GenBank accession numbers and region of sequences used for phylogenetic analyses.

Fig. number	Species name	GenBank accession n°	Region	Source
1	*Alloionema appendiculatum*	KJ851581	18S-ITS1-5.8S-ITS2-28S	Nermut' *et al.* (2015)
	Alloionema appendiculatum	KY355082	18S-ITS1-5.8S-ITS2-28S	This study
	Neoalloionema tricaudatum	KR817921	18S-ITS1-5.8S-ITS2-28S	Ivanowa et al. (2016)
	Alloionema sp.	KP204849	18S-ITS1-5.8S-ITS2-28S	Nermut' *et al.* (2015)
	Neoalloionema sp.	KX017496	18S-ITS1-5.8S-ITS2-28S	Unpublished
	Strongyloides procyonis	AB205054	18S-ITS1-5.8S-ITS2-28S	Sato *et al.* (2006)
	Strongyloides fuelleborni	AB272235	18S-ITS1-5.8S-ITS2-28S	Sato *et al.* (2007)
	Strongyloides callosciureus	AB272229	18S-ITS1-5.8S-ITS2-28S	Sato *et al.* (2007)
	Strongyloides robustus	AB272232	18S-ITS1-5.8S-ITS2-28S	Sato *et al.* (2007)
	Rhabditophanes sp.	KP204851	18S-ITS1-5.8S-ITS2-28S	Nermut' *et al.* (2015)
	Steinernema feltiae	AB243439	18S-ITS1-5.8S-ITS2-28S	Kuwata *et al.* (2006)
2	*Angiostoma margaretae*	MF192968	18S-ITS1-5.8S-ITS2	Unpublished
	Angiostoma norvegicum	MK214816	18S-ITS1-5.8S-ITS2	Singh *et al.* (2019)
	Angiostoma gandavensis	MK214815	18S-ITS1-5.8S-ITS2	Singh *et al.* (2019)
	Angiostoma dentiferum	MK214814	18S-ITS1-5.8S-ITS2	Singh *et al.* (2019)
	Phasmarhabditis neopapillosa	FJ516760	ITS1-5.8S-ITS2	Unpublished
	Phasmarhabditis hermaphrodita	FJ516761	ITS1-5.8S-ITS2	Unpublished
	Phasmarhabditis hermaphrodita	KM510202	ITS1-5.8S-ITS2	Tandigan *et al.* (2014)
	Phasmarhabditis hermaphrodita	KY355083	ITS1-5.8S-ITS2	This study
	Agfa flexilis	MK214813	18S-ITS1-5.8S-ITS2	Singh *et al.* (2019)
	Caenorhabditis elegans	FJ589007	18S-ITS1-5.8S-ITS2-28S	Imai *et al.* (2009)
3	*Angiostoma margaretae*	HQ115062	18S	Ross *et al.* (2011)
	Angiostoma sp.	KY355084	18S	This study
	Angiostoma norvegicum	KU712560	18S	Ross *et al.* (2017)
	Angiostoma dentiferum	MK214806	18S	Singh *et al.* (2019)
	Phasmarhabditis hermaphrodita	FJ516755	18S	Ross *et al.* (2010b)
	Phasmarhabditis neopapillosa	FJ516754	18S	Ross *et al.* (2010b)
	Phasmarhabditis californica	KM510210	18S	Tandigan *et al.* (2014)
	Angiostoma dentifera	FJ516752	18S	Ross *et al.* (2010b)
	Phasmarhabditis papillosa	KM510211	18S	Tandigan *et al.* (2014)
	Oscheius chongmingensis	EU273597	18S	Liu *et al.* (2012)
	Oscheius insectivora	AF083019	18S	Unpublished (2002)
	Pellioditis marina	AF083021	18S	Unpublished
	Angiostrongylus vasorum	AJ920365	18S	Chilton *et al.* (2006)
	Heterorhabditis indica	LN611143	18S	Unpublished
	Steinernema feltiae	FJ381667	18S	Unpublished
4	*Rhabdias bufonis*	KF999593	ITS1-5.8S-ITS2-28S	Tkach *et al.* (2014)
	Rhabdias engelbrechti	MG428406	18S-ITS1-5.8S-ITS2-28S	Kuzmin *et al.* (2017)

Fig. number	Species name	GenBank accession n°	Region	Source
	Rhabdias bulbicauda	KF999600	ITS1-5.8S-ITS2-28S	Tkach *et al.* (2014)
	Rhabdias bermani	KF999610	ITS1-5.8S-ITS2-28S	Tkach *et al.* (2014)
	Rhabdias elegans	KF999604	ITS1-5.8S-ITS2-28S	Tkach *et al.* (2014)
	Rhabdias pseudosphaerocephala	EU836873	ITS1-5.8S-ITS2-28S	Dubey &Shine (2008)
	Rhabdias bakeri	EU360831	18S-ITS1-5.8S-ITS2-28S	Dare *et al.* (2008)
	Rhabdias tarichae	MH023523	18S-ITS1-5.8S-ITS2-28S	Johnson *et al.* (2018)
	Rhabdias picardiae	MG195567	18S-ITS1-5.8S-ITS2-28S	Svitin *et al.* (2018)
	Rhabdias sylvestris	KJ018777	ITS1-5.8S-ITS2-28S	Tkach *et al.* (2014)
	Pneumonema sp.	KF999603	ITS1-5.8S-ITS2-28S	Tkach *et al.* (2014)
	Pneumonema tiliquae	KF999611	ITS1-5.8S-ITS2-28S	Tkach *et al.* (2014)
	Entomelas entomelas	KF999592	ITS1-5.8S-ITS2-28S	Tkach *et al.* (2014)
	Entomelas sp.	KF999601	ITS1-5.8S-ITS2-28S	Tkach *et al.* (2014)
	Entomelas dujardini	KF999591	ITS1-5.8S-ITS2-28S	Tkach *et al.* (2014)
	Entomelas sp.	KY355086	ITS1-5.8S-ITS2-28S	This study
	Steinernema feltiae	AB243439	18S-ITS1-5.8S-ITS2-28S	Kuwata *et al.* (2006)
5	*Leucochloridium paradoxum*	KP903688	ITS1-5.8S-ITS2-28S	Heneberg *et al.* (2016)
	Leucochloridium sp.	AY258145	ITS1-5.8S-ITS2-28S	Casey *et al.* (2003)
	Leucochloridium vogtianum	KP903700	ITS1-5.8S-ITS2-28S	Heneberg *et al.* (2016)
	Leucochloridium perturbatum	KP903707	5.8S-ITS2-28S	Heneberg *et al.* (2016)
	Brachylaima sp.	JX010634	5.8S-ITS2-28S	Unpublished
	Brachylaima mesostoma	KT074964	5.8S-ITS2-28S	Heneberg *et al.* (2016)
	Brachylaima mesostoma	KY355085	5.8S-ITS2-28S	This study
	Clinostomum album	KU708008	18S-ITS1-5.8S-ITS2-28S	Rosser *et al.* (2017)
	Clinostomum marginatum	KU708007	18S-ITS1-5.8S-ITS2-28S	Rosser *et al.* (2017)
	Macroderoides sp.	HQ680850	18S-ITS1-5.8S-ITS2-28S	Tkach & Kinsella (2011)
	Macroderoides texanus	EU850398	18S-ITS1-5.8S-ITS2-28S	Tkach *et al.* (2008)
	Macroderoides flavus	HQ680851	18S-ITS1-5.8S-ITS2-28S	Tkach & Kinsella (2011)
	Dicrocoelium hospes	EF102026	5.8S-ITS2-28S	Maurelli *et al.* (2007)
	Lutztrema attenuatum	KU563718	5.8S-ITS2-28S	Unpublished
	Eurytrema sp.	KY355087	5.8S-ITS2-28S	This study
	Concinnum ten	AB521802	5.8S-ITS2-28S	Sato *et al.* (2010)
	Eurytrema pancreaticum	KY490000	18S-ITS1-5.8S-ITS2-28S	Su *et al.* (2018)
	Opisthorchis viverrini	MG797539	5.8S-ITS2-28S	Sanpool *et al.* (2018)
6	*Raillietina echinobothrida*	MH122787	5.8S-ITS2-28S	Unpublished
	Skrjabinia cesticillus	AY382321	5.8S-ITS2-28S	Unpublished
	Skrjabinia sp.	KY355088	5.8S-ITS2-28S	This study
	Parorchites zederi	KP893424	18S-ITS1-5.8S-ITS2-28S	Kleinertz *et al.* (2014)
	Hymenolepis nana	LC389873	ITS2	Banzai *et al.* (2018)
	Mesocestoides litteratus	MH936660	ITS1-5.8S-ITS2-28S	Unpublished
	Mesocestoides sp.	MH936661	ITS1-5.8S-ITS2-28S	Unpublished
	Proteocephalus torulosus	AY375549	5.8S-ITS2-28S	Scholz *et al.* (2003)

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	Raillietina sp.	MK201802	5.8S-ITS2	Unpublished
	Raillietina beveridgei	AY382318	5.8S-ITS2-28S	Unpublished
	Raillietina dromaius	AY382320	5.8S-ITS2-28S	Unpublished
	Raillietina australis	AY382317	5.8S-ITS2-28S	Unpublished
	Raillietina chiltoni	AY382319	5.8S-ITS2-28S	Unpublished
	Hymenolepis diminuta	MF143799	ITS1-5.8S-ITS2	Unpublished